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TITLE: Lymphatic Reprogramming of Adult Endothelial Stem Cells for a Cell-Based Therapy for Lymphedema in Breast Cancer Patients

PRINCIPAL INVESTIGATOR: Young Kwon Hong, Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
Los Angeles, CA 90033

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14. ABSTRACT This research is designed to develop an efficient method to isolate and culture endothelial cell progenitor/stem cells from human adult blood and further to differentiate them into lymphatic lineage to be eventually used for cell-based therapy for post-operational lymphedema patients. The key significance of our proposal is to utilize the elusive circulating adult stem cells to avoid the ethical and immunological problems that the embryonic stem cell research faces. During the funding period, we made important progresses in isolation and culture of adult endothelial progenitor cells. In particular, we found that mononuclear cells from bloods displayed increased mitogenic potential in the endothelial cell basal medium that contains vascular endothelial cell growth factor (VEGF)-A and VEGF-C. Moreover, we found that addition of interleukin-3 activated the cells to upregulate Prox1, the master control gene for lymphatic development. However, we also found that adult endothelial progenitor cells failed to grow more than three passages under the current condition. We are currently investigating the optimal condition for the long term culture of these cells.					
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Annual Report
Sept 1, 2007 – Aug 31, 2008
Award Number W81XWH-07-1-0539

Introduction:

The object of this study is to develop a technology that allows us to obtain lymphatic endothelial cells (LECs) from patient-derived adult stem cells to rebuild the damaged lymphatic vessels of breast cancer survivors with lymphedema. This patient-derived EPC-based approach will not have the ethical and immunorejection problems that the embryonic stem cell-based therapy face

Body of the Report:

We aimed to isolate endothelial progenitor/stem cells from human blood and to further differentiate into lymphatic endothelial cell lineage as the initial technology development for cell-based therapy. Because the terms and conditions of this award do not allow generation of new human material, we initially utilized the existing bloods from tumor banks. Using the conventional Ficoll density gradient method, we purified and cultured peripheral blood mononuclear cells (PBMCs). Because the isolation and purification method has not been established, our initial goal was to find the optimal culture conditions and we have tried several different culture conditions by changing matrix components and culture medium components. Despite a substantial amount of time and effort to optimize culture condition, the frozen bloods did not yield good quality of PBMCs. However, we found that fibronectin, among tested extracellular matrix (ECM), promotes the best adhesion of the endothelial progenitor/stem cells to culture plates, which may serve an excellent extracellular matrix. Moreover, we found that, compared to the endothelial cell complete media (EBM plus 20% FBS), the commercially available endothelial cell media, EGM-2 (Lonza, Inc) supported a better growth. We then explored other sources such as partially purified blood cells from our collaborator and commercial sources.

The following civil service employees are paid fringe benefits at the rate of 32% year.

- Mr. Jaehyuk Yoo, MS, was hired at a salary of \$33,000/year and has spent 100% of his time for this project.
- Mr. Sathish Kumar Ganesan, MS, has spent 36 % of his time at a salary of \$25,000 for this project.
- Dr. Young-Kwon Hong has spent 9% of his time of \$90,000 for this project.

Key Research Accomplishments:

1. Successful lymphatic reprogramming of progenitor cells from freshly isolated blood by our collaborator

We attributed our failure of isolating endothelial precursor cells from frozen bloods due to a prolonged storage of the blood. In order to explore this possibility, we obtained “fresh” cells from Dr. Marvin Yoder (Indiana University School of Medicine, Indianapolis) to perform a proof-of-concept experiment. The partially purified adherent cells were cultured following our scheme illustrated in **Figure 1**. After culturing the cells according to this new approach, we performed gene expression analysis for two most important lymphatic-specific genes, Prox1 and LYVE-1, by using quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR). This assay demonstrated that after 6-day, but not 3 day incubation, the cells were showing indications that they were successfully reprogrammed into lymphatic lineage cells (**Figure 2**). Despite this success, we were not able to expand the reprogrammed cells enough to perform large-scale *in vitro* characterization. This may be due to low proliferative potential of adult progenitor cells and/or to low number of “true” progenitor cells in adult blood.

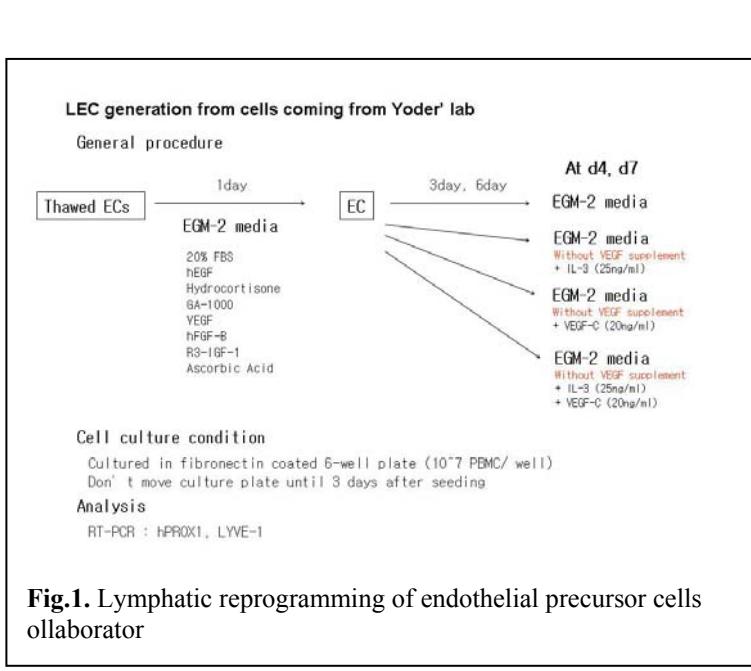


Fig.1. Lymphatic reprogramming of endothelial precursor cells collaborator

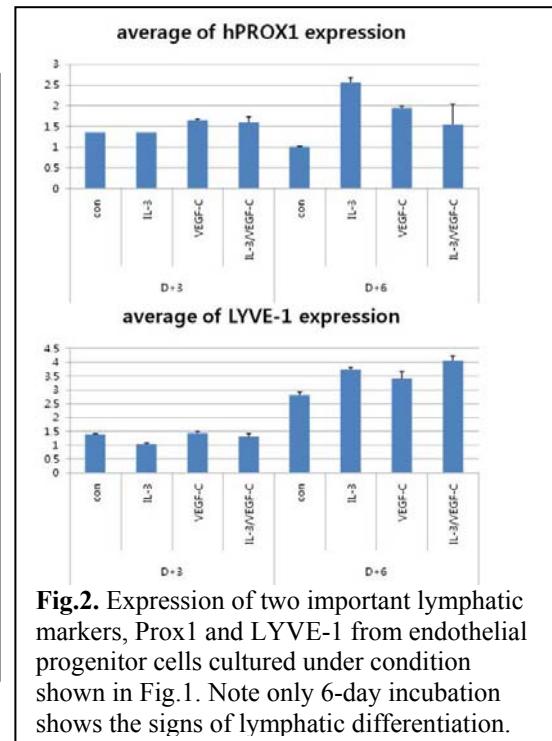


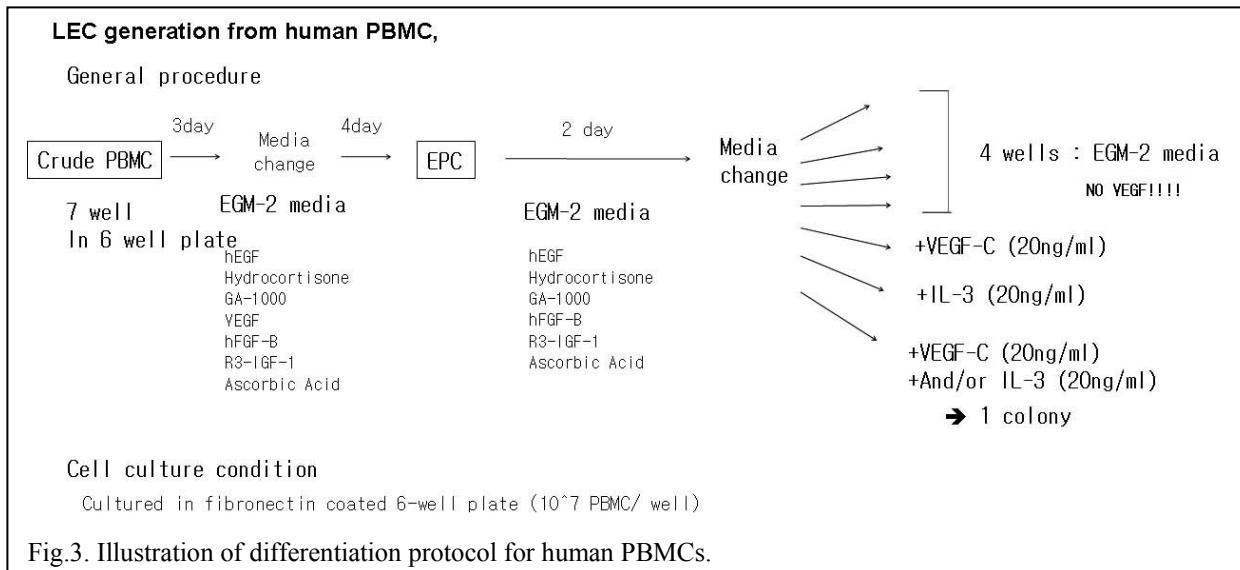
Fig.2. Expression of two important lymphatic markers, Prox1 and LYVE-1 from endothelial progenitor cells cultured under condition shown in Fig.1. Note only 6-day incubation shows the signs of lymphatic differentiation.

2. Successful culture of crude adult PBMC-derived endothelial progenitor cells

We also obtained crude adult human PBMCs from our collaborator, Dr. YongGuen Kwon (Yonsei University, Seoul, South Korea). To improve culture condition, we have further modified our protocol as shown in Figure 3. We found that the crude PMBCs did not adhere and grow well without VEGF-A, and that they require either VEGF-C or IL-3. In particular, IL-3 showed a dramatic improvement in cell growth. Addition of both VEGF-C and IL-3 did not show any difference than IL-3 alone (data not shown). We also found the similar potency from commercially available purified PBMCs.

3. Successful culture of cord blood-derived endothelial progenitor cells

We have also attempted to isolate and culture endothelial cell progenitor cells from crude cord blood cell population provided by our collaborator Dr. Kwon (Yonsei University, Seoul, South Korea). We found that the cord blood crude cells harbor even more population of endothelial progenitor cells and they grow much better even in the absence of IL-3.



The Statement of Work

Task1 Completed We have successfully isolate endothelial precursor cells from peripheral bloods and commercially available purified blood cells. We have tried a number of extracellular matrix proteins, serum concentration and different media compositions. Depending on culture media and coating matrix proteins, endothelial precursor cells displayed dramatic difference in cell viability and growth.

Task2 Completed We have constructed Prox1 expressing vectors. We generated a TAT-Prox1-His fusion construct and produce and purify the recombinant protein from *E. coli* by using a nickel column. We confirmed this by western analysis.

Task3 Completed Endothelial precursor cells were stimulated by IL-3 and lymphatic reprogramming was achieved based on real time RT-PCR to assess the expression of lymphatic markers.

Task4 Completed Prox1 was expressed in endothelial precursor cells and induced lymphatic reprogramming based on real time RT-PCR to assess the expression of lymphatic markers.

Task5 In progress We are now testing the degree of lymphatic reprogramming by various cellular and molecular approaches including microarray, in vitro vessel formation assay, etc.

Task6 Not Initiated In vivo characterization has not been initiated.

Reportable outcomes:

- We were able to successfully grow endothelial progenitor cells from human adult PBMC and cord blood.
- VEGF-C and IL-3 promote cell proliferation and viability of endothelial progenitor cells.

- IL-3 treatment can induce lymphatic reprogramming of cord blood-derived endothelial progenitor cells.

Conclusion:

We made a significant progress in the funding period. In particular, we were able to induce cell fate reprogramming of endothelial progenitor cells to lymphatic lineage. Unfortunately, however, we run into a technical hurdle in optimizing for culturing of endothelial progenitor cells from human adult PBMC. We believe that this technical hurdle can be expected to be overcome by adjusting culture conditions of the primary cells. In this remaining funding period until Sept 30 2009, we will focus on differentiating human adult PBMCs into lymphatic lineages as proposed.

Young Kwon Hong, Ph.D.
Assistant Professor
Departments of Surgery
and of Biochemistry & Molecular Biology
Norris Comprehensive Cancer Center
University of Southern California
1450 Biggy St. NRT6501
M/C9601
Los Angeles, CA 90033
Phone: 323-442-7825
FAX: 323-442-7844
e-mail: young.hong@usc.edu